METHOD FOR MODULATING G PROTEIN COUPLED RECEPTORS

CROSS REFERENCE TO RELATED APPLICATIONS

This application claims the benefit of U.S. Application Serial No. 60/054,165, filed July 30, 1997, and U.S. Application Serial No. 60/054,492, filed August 1, 1997, the disclosures of which are incorporated by reference.

FEDERALLY SPONSORED RESEARCH AND DEVELOPMENT

This invention was made with government support under Grant No. R01 EYO6979, awarded by the National Institutes of Health. The government has certain rights in this invention.

FIELD OF THE INVENTION

The present invention relates to methods of screening for modulators, of G-protein coupled receptor signal transduction activity, and methods for screening for mutant RDGC phosphatases associated with G-protein coupled receptor signal transduction disease.

BACKGROUND OF THE INVENTION

Many physiological signals (e.g., sensory, hormonal, and neurotransmitter signals) are transduced from extracellular to intracellular environments by cell surface receptors. For example, G-protein coupled receptors mediate responses to a wide range of extracellular stimuli, including hormones, neurotransmitter, peptides, and sensory signals such as odorants, light, and taste ("GPCRs"; *see, e.g.*, Neer, *Cell* 80:249-257 (1995); Fong, *Cell Signal.* 8:217 (1996); and Baldwin, *Curr. Opin. Cell Biol.* 6:180 (1994)). These receptors share several structural features including seven putative transmembrane domains and the presence of clustered serine and threonine residues in the cytoplasmic carboxyl terminus of the molecule.

GPCRs mediate signal transduction across a cell membrane upon activation, e.g., the binding of a ligand to an extracellular portion of the GPCR or activation by other stimulus such as light. The intracellular portion of a GPCR interacts with a G-protein to modulate signal transduction from outside to inside a cell. A GPCR is therefore said to be "coupled" to a G-protein. The effects of bacterial toxins that target G proteins for covalent modification signal the potential importance of G protein dysfunction as a cause of human disease.

G-proteins are heterotrimers composed of three polypeptide subunits: an α subunit, which binds and hydrolyzes GTP, and a dimeric $\beta\gamma$ subunit. In the basal, inactive state, the G-protein exists as a heterotrimer of the α and $\beta\gamma$ subunits. When the G-protein is inactive, guanosine diphosphate (GDP) is associated with the α subunit of the G-protein. When a GPCR is bound and activated, e.g., by a ligand, the GPCR binds to the G-protein and decreases the affinity of the Gα subunit for GDP. In its active state, the Gα subunit exchanges GDP for guanine triphosphate (GTP) and then active Gα subunit disassociates from both the receptor and the dimeric $\beta\gamma$ subunit. The disassociated, active Gα subunit transduces signals to effectors that are "downstream" in the G-protein signaling pathway within the cell. Such effectors include enzymes like adenylate cyclase and phospholipase C. Eventually, the G-protein's endogenous GTPase activity returns active Gα subunit to its inactive state, in which it is associated with GDP and the dimeric $\beta\gamma$ subunit.

Many GPCRs share desensitization and inactivation mechanisms, such as the β-adrenergic receptor and rhodopsin (*see, e.g.*, Hein & Kobilka, *Neuropharmacology* 34:357 (1995); Lefkowitz *et al., Adv. Second Messenger Phosphoprotein Res.* 28:1 (1993); Nathans, *Biochemistry* 31:4923 (1992); Hargrave & McDowell, *Int. Rev. Cytol.* 137B:49 (1992); and Lagnado & Baylor, *Neuron* 8:995 (1992)). Light or ligand activation results in phosphorylation of the GPCR by a GPCR kinase. Phosphorylation results in a slight decrease in receptor activity, but also causes the receptor to become a high affinity substrate for arrestin. Arrestin binding terminates the active state of the receptor by preventing its coupling to G protein. Receptor dephosphorylation is essential for completion the signaling cycle (*see, e.g.*, Hurley, *Curr. Opin. Neurobiol.* 4:481 (1994); Langlois *et al., Proc. Natl Acad. Sci. USA* 93:4677 (1996); Yang *et al., J. Biol. Chem.* 263:8856 (1988); Fowles *et al., Biochemistry* 28:9385 (1989); Palczewski *et al., Biochemistry* 28:415 (1989); Palczewski *et al., J. Biol. Chem.* 264:15770 (1989); Yang

et al. Biochem Biophys. Res. Commun. 178:1306 (1991); King *et al., Eur. J. Biochem.* 225:383 (1994); and Kutuzov & Bennett, *Eur. J. Biochem.* 238:613 (1996)).

Recently, a gene was isolated which, when mutated, leads to light-dependent retinal degeneration in *Drosophila* (Steele *et al., Cell* 69: 669-676, (1992). The retinal degeneration C gene (rdgC gene, encoding RDGC protein) encodes an unusual type of serine/threonine phosphatase, consisting of an N-terminal domain that has Egh sequence similarity to the catalytic domain of protein phosphatases 1, 2A and 2B, and a C-terminal domain containing multiple EF-hand motifs. Based on its primary structure, RDGC has been proposed to be a calcium regulated phosphatase, and has been shown to be required for efficient dephosphorylation of rhodopsin *in vitro*. (Chen *et al., Science* 267: 374-377, 1995; Byk *et al., Proc. NatL Acad Sci. USA* 90: 1907-1911, 1993). However, the molecular identification of a G protein coupled receptor phosphatase has been difficult. The fact that GPCR's share a number of structural features, including a carboxyl terminal phosphorylation region, indicates that GPCR dephosphorylation is a common theme in controlling receptor function. There is a need to identify mechanisms by which GPCR phosphorylation events can be controlled *in vivo*.

SUMMARY OF THE INVENTION

The present invention thus identifies for the first time a G-protein coupled receptor phosphatase. RDGC phosphatase participates in regulation of G-protein coupled receptor signal transduction by regulating activity of the receptor via desphosphorylation. The invention therefore provides cells and methods for identifying modulators of signal transduction based on GPCR phosphatases that modulate GPCR activity by dephosphorylation. By targeting GPCR phosphatases in the assays, as described herein, modulators of signal transduction can be identified.

The invention includes methods and kits for identifying modulators of GPCR signal transduction comprising contacting a GPCR phosphatase in a biochemical assay, cell assay or animal assay, with a test compound and detecting a functional effect on GPCR mediated signal transduction. As described herein, the invention includes animals (e.g., mice and flies) and cells (e.g., mammalian and insect) comprising GPCR phosphatases, modified GPCR phosphatases and defective GPCR phosphatases to use in assays of signal transduction. The invention also includes proteins (as well as polynucleotides encoding the same) corresponding to GPCR phosphatases, modified

GPCR phosphatases or defective GPCR phosphatases to use in assays of signal transduction.

BRIEF DESCRIPTION OF THE DRAWINGS

FIGURE IA shows an autoradiogram of an SDS-PAGE of $^{32}PO_4$ *in vivo* labeled retinal proteins.

FIGURE 1B shows an autoradiogram of an SDS-PAGE of $^{32}PO_4$ *in vivo*-labeled retinal proteins after a 20 s pulse of light. In the top panel, B refers to flies exposed to 20 s of blue light, BO refers to flies exposed to 20 s of blue light followed by 20 s of orange light. Rhl stands for rhodopsin 1 (36 kD). Rh1D356 is the truncation of the carboxy-terminal tail of rhodopsin. The bottom panel shows the same gel blotted and probed with anti-Rh1 antibodies.

FIGURE 1C shows a histogram of the relative level of ^{32}P incorporation into Rh1 rhodopsin. Samples were normalized to control flies exposed to blue and orange light sequentially (BO) and corrected for amount of rhodopsin loaded. Data are means $\pm$SEM of triplicate determinations.

FIGURE 2A shows a 1 μm thick cross section of a wild type adult retinas after 6 days of light exposure. The retinas display normal retinal morphology, with ommatidial clusters organized in a well patterned array.

FIGURE 2B shows a 1 μm thick cross section of mutant retinas from rdgC flies displaying dramatic retinal degeneration.

FIGURE 2C shows a 1 μm thick cross section of mutant retinas from Dgq mutants which lack the α-subunit of the heterotrimeric G protein that couples rhodopsin to PLC and do not display light-dependent degeneration.

FIGURE 2D shows a 1 μm thick cross section of mutant Dgq retinas indicating that this mutant is unable to suppress the degeneration of the Dgq; rdgC double mutants.

FIGURE 2E shows a 1 μm thick cross section of mutant Rh1D356 transgenic animals displaying normal retinal morphology.

FIGURE 2F shows a 1 μm thick cross section of mutant Rh1D356 which drastically suppresses rdgC degeneration in Rh1D356; rdgC double mutants.

FIGURE 3A shows electroretinograms (ERGS) of w, rdgC, Rh1D356 and Rh1D356; rdgC mutant flies. Photoreceptors were given a 0.5 s pulse of orange light.

rdgC mutants display strong defects in the deactivation kinetics (arrow), and this phenotype is suppressed by the Rh1D356 truncation. Traces from 15 independent measurements were averaged.

FIGURE 3B shows intracellular recordings of light-activated responses from control and rdgC photoreceptors. This recording configuration demonstrates the photoreceptor specificity of the phenotype; note the strong deactivation defect phenotype in rdgC (arrow). For control flies, t50 = 34±2 ms, for rdgC t5O = 136 ± 22 ms (n = 26), data are means ± SEM.

FIGURE 3C shows a histogram of the deactivation time for the different ERG phenotypes. For control, t85 = 0. 17 ± 0.03 s, for rdgC t85 = 1.8 ± 0.3 s, for Rh1D356 t85 = 0. 1 6 ± 0.03 s, for Rh1D356; rdgC t85 = 0.32 ± 0.03 s (n = 15), data represents means ± SD.

FIGURE 4A shows an ERG recording of a white eyed control fly showing a prototypical prolonged depolarizing afterpotential (PDA) response. 0, orange light (M'R conversion); B, blue light (R'M conversion).

FIGURE 4B shows a histogram of relative amounts of blue light required to enter a PDA for the different genotypes. Values are normalized to control flies and represent means ± SEM of triplicate determinations. Arr2, a known mutant with a PDA defect is shown for comparative purposes.

DESCRIPTION OF THE PREFERRED EMBODIMENT

I. Introduction

The present invention provides for the first time the molecular identity of a G-protein coupled receptor phosphatase. RDGC has been identified as a phosphorylase for a GPCR substrate, rhodopsin. The molecular identification of the first GPCR phosphatase provides a means for identifying novel therapeutic agents that modulate GPCR signal transduction. Such agents are useful for treating diseases related to GPCR signal transduction. Such modulators of GPCR signal transduction can be identified using high throughput assay techniques, using the assays described herein.

The invention includes means for identifying modulators of GPCR signal transduction comprising contacting a GPCR phosphatase such as RDGC in a biochemical assay, cell assay or animal assay, with a test chemical and detecting a change in signal transduction. As described herein, the assays of the invention include animals (e.g.,

mice and flies) and cells (e.g., mammalian and insect) comprising GPCR phosphatase, modified GPCR phosphatase, or defective GPCR phosphatase. The invention also includes proteins (as well as polynucleotides encoding the same) corresponding to GPCR phosphatases, modified GPCR phosphatases or defective GPCR phosphatases to use in assays of signal transduction.

The *Drosophila* phototransduction system is described herein as an example of GPCR signal transduction. This phototransduction cascade is a G-protein coupled, PLC-signaling pathway that shares many features with other signaling cascades (see, for example, Ranganathan *et al.*, *Annual Review of Neuroscience,* 18:283-317 (1995), which is incorporated herein by reference). Light activation of rhodopsin activates a Gqα (a G-protein), which in turn activates a PLC-β (phospholipase C) (see, for example, Pak, *Invest. Ophthalmol. Vis. Sci.*, 36:2340-2357 (1995), which is incorporated herein by reference). PLC_β catalyzes the breakdown of phosphatidylinositol bisphosphate (PIP_2) into the two intracellular messengers inositol triphosphate (IP_3) and diacylglycerol (DAG), which leads to the eventual opening (and modulation) of the TRP and TRPL light-activated channels (see, for example, Niemyer *et al.*, *Cell,* 85:651-659 (1996), which is incorporated herein by reference). Following termination of the stimulus, calcium-dependent regulatory processes, including activation of eye-PKC (protein kinase C), mediate deactivation of the light response (see, for example, Smith *et al.*, *Science*, 254:1478-1484 (1991), which is incorporated herein by reference).

InaD is one of the many loci that have been identified in genetic screens designed to dissect this signaling cascade. A single mutant allele, $InaD^{215}$ photoreceptor deactivation (see, for example, Pak, *Mutants affecting the vision in Drosophila melaogaster* 1-703-733 (Plenum, New York/London, 1975); Shieh *et al.*, *Neuron,* 14:201-210 (1995), which are incorporated herein by reference). The INAD protein was shown to contain at least two PDZ domains (see, for example, Shieh *et al.*, (1995), supra), and to interact with the TRP ion channel (see, for example,, Shieh & Zhu, *Neuron*, 16:991-998 (1996). More recently, several groups reported that INAD can be found associated with multiple components of the phototransduction cascade, including PLC_β, eye-PKC, rhodopsin and calmodulin (see, for example, Shieh and Zhu, (1996), supra; Huber et al., *Embo J,* 15:7036-7045 (19996); and Chevesich et al., *Neuron,* 18:95-105 (1997), each of which is incorporated herein by reference).

In one embodiment of the present application, phototransduction in *Drosophila* was used as a model to study the activation and deactivation of GPCRs *in vivo* and *in vitro* (see Examples 1-4). In this signaling pathway, photoreceptor neurons report activity with exquisite sensitivity and specificity. In addition, photoreceptors achieve superb temporal resolution by ensuring that the transduction machinery is reset quickly after generating a response. Phototransduction in *Drosophila* is the fastest known G protein-coupled cascade, taking just a few tens of milliseconds to go from light activation of rhodopsin to the generation of a receptor potential, and less than 100 ms to shut-off following termination of the stimulus (see, for example, Rancanathan *et al.*, (1995), *supra*). This model of signal transduction, as well as other examples of *Drosophila* biology, applies to signal transduction in humans.

The invention also includes test compounds identified by the methods described herein. For example, the invention includes chemicals identified by screening for the activity of a RDGC phosphatase.

The present invention also relates to the use of test compounds identified using the methods described herein to treat GPCR associated diseases. As used herein, a "G protein-coupled receptor associated disorder" refers to a disorder characterized by the failure of a cell(s) of a mammal to produce a level of a wild-type G-protein comparable to the level produced in a mammal not having the disorder. Such disorders include those in which a G-protein(s) is absent (e.g., as the result of a null mutation), produced at low levels, or dysfunctional (e.g., as the result of a point mutation, deletion, or insertion). Examples of such disorders include color blindness, nephrogenic diabetes insipidus, familial ACTH resistance, familial hypocalciuric hypercalcimia, congenital night blindness, sporadic hyperfunctional thyroid nodules, congenital bleeding, Jansen metaphyseal chondrodysplasia, Hirschspruno, disease, familial hypothyroidism, Albright hereditary osteodystrophy, McCune-Albright syndrome, alcoholism, diabetes mellitus and hypertension. Accordingly, treatment of the disorder entails expressing in a cell(s) of the mammal a polypeptide having the biological activity of a promiscuous G-protein; the polypeptide thus compensates for the abnormal endogenous G-protein.

II. Definitions

Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to

which this invention belongs. Generally, the nomenclature used herein and the laboratory procedures in spectroscopy, drug discovery, cell culture, and molecular genetics, described below are those well known and commonly employed in the art. Standard techniques are typically used for preparation of signal detection, recombinant nucleic acid methods, polynucleotide synthesis, and microbial culture and transformation (e.g., electroporation, and lipofection). The techniques and procedures are generally performed according to conventional methods in the art and various general references (see generally, Sambrook et al. Molecular Cloning: A Laboratory Manual, 2d ed. (1989) Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., and Lakowicz, J.R. *Principles of Fluorescence Spectroscopy,* New York: Plenum Press (1983) for fluorescence techniques, which are incorporated herein by reference) which are provided throughout this document. Standard techniques are used for chemical syntheses, chemical analyses, and biological assays. As employed throughout the disclosure, the following terms, unless otherwise indicated, shall be understood to have the following meanings:

"RDGC phosphatase" is a protein that has phosphatase activity for G-protein coupled receptors. As used herein, the term "RDGC phosphatase" includes naturally occurring RDGC, recombinant RDGC, and functional RDGC equivalents that have GPCR phosphatase activity, e.g., interspecies homologs, alleles, polymorphic variants and the like. The term RDGC therefore refers to RDGC homologs such as polymorphic variants, alleles, mutants, and closely related interspecies variants that have about at least 60% amino acid sequence identity to RDGC phosphatase (e.g., are substantially identical to RDGC; see the sequence of RDGC in Steele *et al., Cell* 69:669-676 (1992), herein incorporated by reference) and have GPCR phosphatase activity, as determined using the assays described herein.

"RDGC GPCR phosphatase activity" is the activity of an RDGC phosphatse for a he GPCR substrate. In addition to RDGC, other GPCR phosphatases have the same activity for a GPCR substrate. This activity can be measured with endogenously or recombinantly expressed RDGC or GPCR phosphatases and signal transduction proteins using the assays described herein. GPCR and RDGC phosphatases permit enhanced signal transduction by signal transduction proteins compared to signal transduction in the absence of a GPCR. Mammalian GPCR phosphatases for use with

mammalian signal transduction proteins are preferred, particularly human GPCR phosphatases.

"GPCR phosphatase" refers to a protein with the GPCR phosphatase activity of RDGC phosphatase or another structurally similar phosphatase regulating the phosphorylation state of a G-protein coupled receptor phosphatase protein. Such phosphatases can regulate G-protein coupled receptor phosphorylation levels which in turn regulates signal transduction activity and its associated proteins. Examples of signal transduction-proteins include GPCRS, tyrosine kinase receptors, tyrosine phosphatase receptors, ion channels, phospholipases, adenylate cyclases, kinases and G-proteins.

An "RDGC mimetic" refers to a compound or molecule, e.g., a peptide, polypeptide, or small chemical molecule, that recognizes GPCR as a substrate and dephosphorylates a GPCR at the same site as RDGC. RDGC mimetics thus include RDGC homologs. RDGC mimetics would also include small RDGC peptides that retained the RDGC active site, and conservatively modified variants thereof, as well as truncated versions of RDGC that retained RDGC phosphatase activity. Small chemical molecules that mimic the RDGC active site are also RDGC mimetics. RDGC mimetics are tested using assays for RDGC activity, e.g., rhodopsin mobility assays, and rhodopsin phosphorylation, as described below. When testing for an RDGC mimetic, wild-type RDGC is typically used as a positive control for RDGC activity. A relative activity value is assigned to RDGC, e.g., 100. Mimetic activity is achieved when mimetic RDGC activity relative to the control is about 25, more preferably 50-100.

A "G-protein coupled receptor" or "GPCR" is a receptor with seven transmembrane regions. The GPCR has "G-protein coupled receptor activity," e.g., it binds to G-proteins in response to extracellular stimuli and promotes production of second messengers such as IP3, cAMP, and Ca^{2+} via stimulation of enzymes such as phospholipase C and adenylate cyclase (for a description of the structure and function of GPCRs, *see, e.g.*, Fong, *supra*, and Baldwin, *supra*).

"Sample" as used herein is a sample of tissue, cells, membranes, or fluid in aqueous solution or solid phase or a combination of both that comprises a GPCR and RDGC phosphatase or a nucleic acid encoding a GPCR or RDGC phosphatase. Such samples include, but are not limited to, samples isolated from humans, mice, rats, *Drosophila*. Biological samples may also include sections of tissues such as frozen sections taken for histological purposes. A biological sample is typically obtained from

a eukaryotic organism, such as insects, protozoa, birds, fish, reptiles, and preferably a mammal such as rat, mouse, cow, dog, guinea pig, or rabbit, and most preferably a primate such as chimpanzees or humans.

An "animal" refers to any non-human animal.

The term "detecting" refers to the means by which RDGC phosphatase activity is identified. The term thus encompasses any assay, direct or indirect, used to ascertain the presence or absence or level of RDGC phosphatase activity in a sample or animal. Typically detection of RDGC phosphatase activity involves a determination of any parameter that is indirectly or directly under the influence of RDGC phosphatase activity and its role as the phosphatase for a GPCR substrate, e.g., any parameter that has a functional effect on GPCR signal transduction. Such assays include changes in ion flux, membrane potential, voltage, conductance, current flow, transcription, signal transduction, receptor-ligand interactions, second messenger concentrations (e.g., cAMP, IP3, or intracellular Ca^{2+}), patch clamping, voltage-sensitive dyes, whole cell currents, radioisotope efflux, inducible markers, oocyte expression; tissue culture cell expression; ligand binding assays; *in vitro*, *in vivo*, and *ex vivo* and also includes other physiologic effects such increases or decreases of neurotransmitter or hormone release. Any suitable label or means of detection can be used in the above assays. A preferred label or detectable moiety in the above assays is a fluorescent label, which is detected by an "detector" apparatus designed to detect fluorescence.

"Inhibitors," "activators," and "modulators" of RDGC phosphatase activity refer to inhibitory or activating molecules identified using *in vitro* and *in vivo* assays for that have a functional effect on GPCR signal transduction as described above, e.g., ligands, agonists, antagonists, and their homologs and mimetics. Inhibitors are compounds that decrease, block, prevent, delay activation, inactivate, desensitize, or down regulate signal transduction, e.g., antagonists. Activators are compounds that increase, open, activate, facilitate, enhance activation, sensitize or up regulate signal transduction, e.g., agonists. Modulators include genetically modified versions of RDGC phosphatase, e.g., with altered activity, as well as naturally occurring and synthetic ligands, antagonists, agonists, small chemical molecules and the like. Such assays for inhibitors and activators include, e.g, expressing RDGC phosphatase in cells or cell membranes, applying putative modulator compounds, and then determining the functional effects on taste transduction, as described above. Samples or assays comprising RDGC

phosphatase that are treated with a potential activator, inhibitor, or modulator are compared to control samples without the inhibitor, activator, or modulator to examine the extent of inhibition. Control samples (untreated with inhibitors) are assigned a relative RDGC phosphatase activity value of 100%. Inhibition of RDGC phosphatase is achieved when the RDGC phosphatase activity value relative to the control is about 90%, preferably 50%, more preferably 25%. Activation of RDGC phosphatase is achieved when the RDGC phosphatase activity value relative to the control is 110%, more preferably 150%, more preferable 200% higher.

"Modulators of GPCR signal transduction" refers to activating or inhibitory molecules identified using *in vitro* and *ex vivo* assays for RDGC phosphatase activity, which in turn have a functional effect on GPCR signal transduction. Potential modulators include peptides, polypeptides, small chemical molecules, synthetic molecule (e.g., nucleic acid, protein, non-peptide, or organic molecules), or an extract made from biological materials such as bacteria, plants, fungi, or animal (particularly mammalian) cells or tissues.

The term "test compound" refers to a compound to be tested by one or more screening method(s) of the invention as a putative modulator. The term "test compound" also does not include chemicals known to be unsuitable for a therapeutic use for a particular indication due to toxicity of the subject. Usually, various predetermined concentrations are used for screening test compounds, such as .01 μM, .1 μM, 1.0 μM, and 10.0 μM.

"Signal transduction" refers to the coupling of an extracellular signal (the signal) to an intracellular response (the response); this term also refers to the response or individual steps of the response. The response need not occur intracellularly, it may also be measured in an non-cellular reaction. Similarly, the signal can also be measured in a non-cellular reaction. For example, an activity directly associated with signal transduction is the activity or physical property of either the receptor (e.g., GPCR), or a coupling protein (e.g., a Gα protein). Signal transduction detection systems for monitoring an activity directly associated with signal transduction, include GTPase activity, ion channel activity and conformational changes. An activity indirectly associated with signal transduction is the activity produced by a molecule other than by either the receptor (e.g., GPCRs, tyrosine kinase receptors, or tyrosine phosphatase receptors), ion channel or a coupling protein (e.g., a Gα protein) associated with a

receptor (e.g., GPCRs, tyrosine kinase receptors, or tyrosine phosphatase receptors), or a coupling protein (e.g., a Gα protein). Such indirect activities include changes in intracellular levels of molecules (e.g., ions (e.g., Ca, Na or K), second messenger levels (e.g., CAMP, cGMP and inositol phosphate)), kinase activities, transcriptional activity, enzymatic activity, phospholipase activities, ion channel activities and phosphatase activities. Signal transduction assays are further described in commonly owned U.S. applications by Negulescu et al., 60/020,234, filed June 21, 1996. Signal transduction detection systems for monitoring an activity indirectly associated with signal transduction, include transcriptional-based assays, enzymatic assays, intracellular ion assays and second messenger assays. The signal for signal transduction can be any signal compatible with the assay being used. The signal may be a test chemical itself or a known activator of signal transduction. The activating step can include activating signal transduction with a signal selected from the group consisting of a chemical signal found in blood, a chemical signal found in a synaptic cleft, a chemical signal found in interstitial fluid, light or a chemical signal found in air or other signals known the art or developed in the future.

A "GPCR mobility assay" refers to an assay for RDGC GPCR phosphatase activity that tests for changes in GPCR electrophoretic mobility after treatment with RDGC and optionally an inhibitor.

A "GPCR phosphorylation assay" refers to an assay for RDGC GPCR phosphatase activity that tests for dephosphorylation after treatment with RDGC.

"A signal transduction assay" refers to any assay that detects GPCR signal transduction, including changes in ion flux, membrane potential, voltage, conductance, current flow, transcription, signal transduction, receptor-ligand interactions, second messenger concentrations (e.g., cAMP, IP3, or intracellular Ca^{2+}), patch clamping, voltage-sensitive dyes, whole cell currents, radioisotope efflux, inducible markers, oocyte expression; tissue culture cell expression; ligand binding assays; *in vitro*, *in vivo*, and *ex vivo* and also includes other physiologic effects such increases or decreases of neurotransmitter or hormone release.

The phrase "contacting a cell" or "contacting a sample" refers to any method whereby RDGC or an RDGC mimetic or homolog is introduced into a cell or sample, e.g., by transduction of a nucleic acid encoding RDGC or an RDGC homolog or mimetic, by administering RDGC to the cell medium or sample, by injecting RDGC or

an RDGC homolog or mimetic into the cell or sample, by conjugating RDGC or an RDGC homolog or mimetic to a molecule, e.g., a receptor ligand, that allows RDGC or the RDGC homolog or mimetic to be translocated into a cell or sample, and by introducing RDGC or the RDGC homolog or mimetic into a cell or sample using a vehicle such as a liposome.

"Transduction" refers to any method whereby a nucleic acid is introduced into a cell, e.g., by transfection, electroporation, passive uptake, lipid:nucleic acid complexes, viral vector transduction, injection, and the like.

An "expression vector" is a nucleic acid construct, generated recombinantly or synthetically, with a series of specified nucleic acid elements that permit transcription of a particular nucleic acid in a host cell. The expression vector can be part of a plasmid, virus, or nucleic acid fragment. Typically, the expression vector includes a nucleic acid to be transcribed operably linked to a promoter.

"*In vivo*" refers to assays that are performed using a whole non-human animal.

"*In vitro*" refers to assays that do not require the presence of a whole animal, e.g., that are performed using a cell, membrane, tissue sample, fluid sample, protein, expression vector, e.g., explants, cultured cell lines, transformed cell lines, primary cell lines, insect eyes, insect heads, extracted tissue, e.g., blood, oocytes, tissue slices, recombinant or naturally occurring proteins, expression vectors encoding proteins, and the like. *In vitro* assays are typically performed in aqueous solution, or with at least one component of the assay bound covalently or non-covalently to a solid phase such as a plate, microchip, bead, column, membrane, dipstick, etc.

"Fluorescent donor moiety" refers to the radical of a fluorogenic compound, which can absorb energy and is capable of transferring the energy to another fluorogenic molecule or part of a compound. Suitable donor fluorogenic molecules include, but are not limited to, coumarins and related dyes xanthene dyes such as fluoresceins, rhodols, and rhodamines, resorufins, cyanine dyes, bimanes, acridines, isoindoles, dansyl dyes, aminophthalic hydrazides such as luminol and isoluminol derivatives, aminophthalimides, aminonaphthalimides, aminobenzofurans, aminoquinolines, dicyanohydroquinones, and europium and terbium complexes and related compounds.

"Quencher" refers to a chromophoric molecule or part of a compound, which is capable of reducing the emission from a fluorescent donor when attached to the donor. Quenching may occur by any of several mechanisms including fluorescence resonance energy transfer, photoinduced electron transfer, paramagnetic enhancement of formation of dark complexes.

"Acceptor" refers to a quencher that operates via fluorescence resonance energy transfer. Many acceptors can re-emit the transferred as energy as fluorescence. Examples include coumarins and related fluorophores, xanthenes such as fluoresceins, rhodols, and rhodamines, resorufins, cyanines, difluoroboradiazaindacenes, and phthalocyanines. Other chemical classes of acceptors generally do not re-emit the transferred energy. Examples include indigos, benzoquinones, anthraquinones, azo compounds, nitro compounds, indoanilines, di- and triphenylrnethanes.

"Binding pair" refers to two moieties (e.g. chemical or biochemical) that have an affinity for one another. Examples of binding pairs include antigen/antibodies, lectin/avidin, target polynucleotide/probe oligonucleotide, antibody/antiantibody, receptor/ligand, enzyme/ligand and the like. "One member of a binding pair" refers to one moiety of the pair, such as an antigen or ligand.

"Dye" refers to a molecule or part of a compound that absorbs specific frequencies of light, including but not limited to ultraviolet light. The terms "dye" and chromophore" are synonymous.

"Fluorophore" refers to a chromophore that fluoresces.

"Membrane-permeant derivative" refers a chemical derivative of a compound that has enhanced membrane permeability compared to an underivativized compound. Examples include ester, ether and carbamate derivatives. These derivatives are made better able to cross cell membranes, i.e. membrane permeant, because hydrophilic groups are masked to provide more hydrophobic derivatives. Also, masking groups are designed to be cleaved from a precursor (e.g., fluorogenic substrate precursor) within the cell to generate the derived substrate intracellularly. Because the substrate is more hydrophilic than the membrane permeant derivative it is now trapped within the cells.

"Nucleic acid" refers to deoxyribonucleotides or ribonucleotides and polymers thereof in either single- or double-stranded form. The term encompasses nucleic acids containing known nucleotide analogs or modified backbone residues or

linkages, which are synthetic, naturally occurring, and non-naturally occurring, which have similar binding properties as the reference nucleic acid, and which are metabolized in a manner similar to the reference nucleotides. Examples of such analogs include, without limitation, phosphorothioates, phosphoramidates, methyl phosphonates, chiral-methyl phosphonates, 2-O-methyl ribonucleotides, peptide-nucleic acids (PNAs).

Unless otherwise indicated, a particular nucleic acid sequence also implicitly encompasses conservatively modified variants thereof (e.g., degenerate codon substitutions) and complementary sequences, as well as the sequence explicitly indicated. The term nucleic acid is used interchangeably with gene, cDNA, mRNA, oligonucleotide, and polynucleotide.

The terms "polypeptide," "peptide" and "protein" are used interchangeably herein to refer to a polymer of amino acid residues. The terms apply to amino acid polymers in which one or more amino acid residue is an artificial chemical mimetic of a corresponding naturally occurring amino acid, as well as to naturally occurring amino acid polymers and non-naturally occurring amino acid polymer.

The term "amino acid" refers to naturally occurring and synthetic amino acids, as well as amino acid analogs and amino acid mimetics that function in a manner similar to the naturally occurring amino acids. Naturally occurring amino acids are those encoded by the genetic code, as well as those amino acids that are later modified, e.g., hydroxyproline, γ-carboxyglutamate, and O-phosphoserine. Amino acid analogs refers to compounds that have the same basic chemical structure as a naturally occurring amino acid, i.e., an α carbon that is bound to a hydrogen, a carboxyl group, an amino group, and an R group., e.g., homoserine, norleucine, methionine sulfoxide, methionine methyl sulfonium Such analogs have modified R groups (e.g., norleucine) or modified peptide backbones, but retain the same basic chemical structure as a naturally occurring amino acid. Amino acid mimetics refers to chemical compounds that have a structure that is different from the general chemical structure of an amino acid, but that functions in a manner similar to a naturally occurring amino acid.

Amino acids may be referred to herein by either their commonly known three letter symbols or by the one-letter symbols recommended by the IUPAC-IUB Biochemical Nomenclature Commission. Nucleotides, likewise, may be referred to by their commonly accepted single-letter codes.

"Conservatively modified variants" applies to both amino acid and nucleic acid sequences. With respect to particular nucleic acid sequences, conservatively modified variants refers to those nucleic acids which encode identical or essentially identical amino acid sequences, or where the nucleic acid does not encode an amino acid sequence, to essentially identical sequences. Specifically, degenerate codon substitutions may be achieved by generating sequences in which the third position of one or more selected (or all) codons is substituted with mixed-base and/or deoxyinosine residues (Batzer *et al.*, *Nucleic Acid Res.* 19:5081 (1991); Ohtsuka *et al.*, *J. Biol. Chem.* 260:2605-2608 (1985); Rossolini *et al.*, *Mol. Cell. Probes* 8:91-98 (1994)). Because of the degeneracy of the genetic code, a large number of functionally identical nucleic acids encode any given protein. For instance, the codons GCA, GCC, GCG and GCU all encode the amino acid alanine. Thus, at every position where an alanine is specified by a codon, the codon can be altered to any of the corresponding codons described without altering the encoded polypeptide. Such nucleic acid variations are "silent variations," which are one species of conservatively modified variations. Every nucleic acid sequence herein which encodes a polypeptide also describes every possible silent variation of the nucleic acid. One of skill will recognize that each codon in a nucleic acid (except AUG, which is ordinarily the only codon for methionine, and TGG, which is ordinarily the only codon for tryptophan) can be modified to yield a functionally identical molecule. Accordingly, each silent variation of a nucleic acid which encodes a polypeptide is implicit in each described sequence.

As to amino acid sequences, one of skill will recognize that individual substitutions, deletions or additions to a nucleic acid, peptide, polypeptide, or protein sequence which alters, adds or deletes a single amino acid or a small percentage of amino acids in the encoded sequence is a "conservatively modified variant" where the alteration results in the substitution of an amino acid with a chemically similar amino acid. Conservative substitution tables providing functionally similar amino acids are well known in the art. Such conservatively modified variants are in addition to and do not exclude polymorphic variants, interspecies homologs, and alleles of the invention.

The following six groups each contain amino acids that are conservative substitutions for one another:

1) Alanine (A), Serine (S), Threonine (T);

2) Aspartic acid (D), Glutamic acid (E);

3) Asparagine (N), Glutamine (Q);

4) Arginine (R), Lysine (K);

5) Isoleucine (I), Leucine (L), Methionine (M), Valine (V); and

6) Phenylalanine (F), Tyrosine (Y), Tryptophan (W).

(*see, e.g.*, Creighton, *Proteins* (1984)).

A "detectable moiety" or label" is a composition detectable by spectroscopic, photochemical, biochemical, immunochemical, or chemical means. For example, useful labels include ^{32}P, fluorescent dyes and molecules, electron-dense reagents, enzymes (e.g., as commonly used in an ELISA), biotin, digoxigenin, or haptens and proteins for which antisera or monoclonal antibodies are available. Preferred detectable moieties or labels are fluorescent dyes and molecules.

A protein that is "linked to a detectable moiety" is one that is bound, either covalently, through a linker, or through ionic, van der Waals or hydrogen bonds to a label such that the presence of the protein may be detected by detecting the presence of the label or detectable moiety bound to the protein.

The term "recombinant" when used with reference, e.g., to a cell, or nucleic acid, protein, or vector, indicates that the cell, nucleic acid, protein or vector, has been modified by the introduction of a heterologous nucleic acid or protein or the alteration of a native nucleic acid or protein, or that the cell is derived from a cell so modified. Thus, for example, recombinant cells express genes that are not found within the native (non-recombinant) form of the cell or express native genes that are otherwise abnormally expressed, under expressed or not expressed at all.

The term "heterologous" when used with reference to portions of a nucleic acid indicates that the nucleic acid comprises two or more subsequences that are not found in the same relationship to each other in nature. For instance, the nucleic acid is typically recombinantly produced, having two or more sequences from unrelated genes arranged to make a new functional nucleic acid, e.g., a promoter from one source and a coding region from another source. Similarly, a heterologous protein indicates that the protein comprises two or more subsequences that are not found in the same relationship to each other in nature (e.g., a fusion protein).

A "promoter" is defined as an array of nucleic acid control sequences that direct transcription of a nucleic acid. As used herein, a promoter includes necessary nucleic acid sequences near the start site of transcription, such as, in the case of a

polymerase II type promoter, a TATA element. A promoter also optionally includes distal enhancer or repressor elements, which can be located as much as several thousand base pairs from the start site of transcription. A "constitutive" promoter is a promoter that is active under most environmental and developmental conditions. An "inducible" promoter is a promoter that is active under environmental or developmental regulation. The term "operably linked" refers to a functional linkage between a nucleic acid expression control sequence (such as a promoter, or array of transcription factor binding sites) and a second nucleic acid sequence, wherein the expression control sequence directs transcription of the nucleic acid corresponding to the second sequence.

An "expression vector" is a nucleic acid construct, generated recombinantly or synthetically, with a series of specified nucleic acid elements that permit transcription of a particular nucleic acid in a host cell. The expression vector can be part of a plasmid, virus, or nucleic acid fragment. Typically, the expression vector includes a nucleic acid to be transcribed operably linked to a promoter.

The terms "identical" or percent "identity," in the context of two or more nucleic acids or polypeptide sequences, refer to two or more sequences or subsequences that are the same or have a specified percentage of amino acid residues or nucleotides that are the same, when compared and aligned for maximum correspondence over a comparison window, as measured using one of the following sequence comparison algorithms or by manual alignment and visual inspection. This definition also refers to the complement of a test sequence, which has a designated percent sequence or subsequence complementarity when the test sequence has a designated or substantial identity to a reference sequence. For example, a designated amino acid percent identity of 70% refers to sequences or subsequences that have at least about 70% amino acid identity when aligned for maximum correspondence over a comparison window as measured using one of the following sequence comparison algorithms or by manual alignment and visual inspection. Preferably, the percent identity exists over a region of the sequence that is at least about 25 amino acids in length, more preferably over a region that is 50 or 100 amino acids in length.

When percentage of sequence identity is used in reference to proteins or peptides, it is recognized that residue positions that are not identical often differ by conservative amino acid substitutions, where amino acids residues are substituted for other amino acid residues with similar chemical properties (e.g., charge or

hydrophobicity) and therefore do not change the functional properties of the molecule. Where sequences differ in conservative substitutions, the percent sequence identity may be adjusted upwards to correct for the conservative nature of the substitution. Means for making this adjustment are well known to those of skill in the art. Typically this involves scoring a conservative substitution as a partial rather than a full mismatch, thereby increasing the percentage sequence identity. Thus, for example, where an identical amino acid is given a score of 1 and a non-conservative substitution is given a score of zero, a conservative substitution is given a score between zero and 1. The scoring of conservative substitutions is calculated according to, e.g., the algorithm of Meyers & Miller, *Computer Applic. Biol. Sci.* 4:11-17 (1988) e.g., as implemented in the program PC/GENE (Intelligenetics, Mountain View, California, USA)..

For sequence comparison, typically one sequence acts as a reference sequence, to which test sequences are compared. When using a sequence comparison algorithm, test and reference sequences are entered into a computer, subsequence coordinates are designated, if necessary, and sequence algorithm program parameters are designated. Default program parameters can be used, or alternative parameters can be designated. The sequence comparison algorithm then calculates the percent sequence identity for the test sequence(s) relative to the reference sequence, based on the designated or default program parameters.

A "comparison window", as used herein, includes reference to a segment of any one of the number of contiguous positions selected from the group consisting of from 25 to 600, usually about 50 to about 200, more usually about 100 to about 150 in which a sequence may be compared to a reference sequence of the same number of contiguous positions after the two sequences are optimally aligned. Methods of alignment of sequences for comparison are well-known in the art. Optimal alignment of sequences for comparison can be conducted, e.g., by the local homology algorithm of Smith & Waterman, *Adv. Appl. Math.* 2:482 (1981), by the homology alignment algorithm of Needleman & Wunsch, *J. Mol. Biol.* 48:443 (1970), by the search for similarity method of Pearson & Lipman, *Proc. Nat'l. Acad. Sci. USA* 85:2444 (1988), by computerized implementations of these algorithms (GAP, BESTFIT, FASTA, and TFASTA in the Wisconsin Genetics Software Package, Genetics Computer Group, 575 Science Dr., Madison, WI), or by manual alignment and visual inspection (*see, e.g.*, Ausubel *et al., supra*).

One example of a useful algorithm is PILEUP. PILEUP creates a multiple sequence alignment from a group of related sequences using progressive, pairwise alignments to show relationship and percent sequence identity. It also plots a tree or dendogram showing the clustering relationships used to create the alignment. PILEUP uses a simplification of the progressive alignment method of Feng & Doolittle, *J. Mol. Evol.* 35:351-360 (1987). The method used is similar to the method described by Higgins & Sharp, *CABIOS* 5:151-153 (1989). The program can align up to 300 sequences, each of a maximum length of 5,000 nucleotides or amino acids. The multiple alignment procedure begins with the pairwise alignment of the two most similar sequences, producing a cluster of two aligned sequences. This cluster is then aligned to the next most related sequence or cluster of aligned sequences. Two clusters of sequences are aligned by a simple extension of the pairwise alignment of two individual sequences. The final alignment is achieved by a series of progressive, pairwise alignments. The program is run by designating specific sequences and their amino acid or nucleotide coordinates for regions of sequence comparison and by designating the program parameters. Using PILEUP, a reference sequence is compared to other test sequences to determine the percent sequence identity relationship using the following parameters: default gap weight (3.00), default gap length weight (0.10), and weighted end gaps. PILEUP can be obtained from the GCG sequence analysis software package, e.g, version 7.0 (Devereaux *et al., Nuc. Acids Res.* 12:387-395 (1984).

Another example of algorithm that is suitable for determining percent sequence identity (i.e., substantial similarity or identity) is the BLAST algorithm, which is described in Altschul *et al., J. Mol. Biol.* 215:403-410 (1990). Software for performing BLAST analyses is publicly available through the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/). This algorithm involves first identifying high scoring sequence pairs (HSPs) by identifying short words of length W in the query sequence, which either match or satisfy some positive-valued threshold score T when aligned with a word of the same length in a database sequence. T is referred to as the neighborhood word score threshold (Altschul *et al, supra*). These initial neighborhood word hits act as seeds for initiating searches to find longer HSPs containing them. The word hits are then extended in both directions along each sequence for as far as the cumulative alignment score can be increased. Cumulative scores are calculated using, for nucleotide sequences, the parameters M (reward score for a pair of

matching residues; always > 0) and N (penalty score for mismatching residues, always < 0). For amino acid sequences, a scoring matrix is used to calculate the cumulative score. Extension of the word hits in each direction are halted when: the cumulative alignment score falls off by the quantity X from its maximum achieved value; the cumulative score goes to zero or below, due to the accumulation of one or more negative-scoring residue alignments; or the end of either sequence is reached. The BLAST algorithm parameters W, T, and X determine the sensitivity and speed of the alignment. The BLASTN program (for nucleotide sequences) uses as defaults a wordlength (W) of 11, an expectation (E) of 10, M=5, N=4, and a comparison of both strands. For amino acid sequences, the BLASTP program uses as default parameters a wordlength (W) of 3, an expectation (E) of 10, and the BLOSUM62 scoring matrix (*see* Henikoff & Henikoff, *Proc. Natl. Acad. Sci. USA* 89:10915 (1989)).

The BLAST algorithm also performs a statistical analysis of the similarity between two sequences (*see, e.g.*, Karlin & Altschul, *Proc. Nat'l. Acad. Sci. USA* 90:5873-5787 (1993)). One measure of similarity provided by the BLAST algorithm is the smallest sum probability (P(N)), which provides an indication of the probability by which a match between two nucleotide or amino acid sequences would occur by chance. For example, a nucleic acid is considered similar to a reference sequence if the smallest sum probability in a comparison of the test nucleic acid to the reference nucleic acid is less than about 0.1, more preferably less than about 0.01, and most preferably less than about 0.001.

An indication that two nucleic acid sequences or polypeptides are substantially identical is that the polypeptide encoded by the first nucleic acid is immunologically cross reactive with the antibodies raised against the polypeptide encoded by the second nucleic acid, as described below. Thus, a polypeptide is typically substantially identical to a second polypeptide, for example, where the two peptides differ only by conservative substitutions. Another indication that two nucleic acid sequences are substantially identical is that the two molecules or their complements hybridize to each other under stringent conditions, as described below.

III. *In vitro* and *in vivo* assays for modulators and mimetics of RDGC phosphatase activity

A. Introduction

The present invention provides assays to identify modulators of RDGC phosphatase and RDGC phosphatase mimetics of this activity. The activity of RDGC phosphatase can be assessed using a variety of *in vitro* and *in vivo* assays, e.g., measuring GPCR phosphorylation, GPCR electrophoretic mobility, current, membrane potential, ion flux, e.g., sodium, measuring sodium concentration, second messengers (e.g., cAMP, IP3, or Ca^{2+}) and transcription levels, neurotransmitter levels, using e.g., voltage-sensitive dyes, radioactive tracers, patch-clamp electrophysiology, transcription assays, and the like. Modulators can also be genetically altered versions of RDGC phosphatase. Such modulators of RDGC phosphatase activity and GPCR signal transduction activity are useful for identifying compounds that can be used to treat disease that are associated with GPCRs.

As a general assay format for RDGC phosphatase modulators, samples or non-human animals that are with treated RDGC phosphatase or RDGC phosphatase homologs or mimetics and with potential RDGC phosphatase inhibitors or activators are compared to control samples or non-human animals without the test compound, to examine the extent of modulation. Modulators of RDGC phosphatase activity are tested using biologically active RDGC phosphatase, either recombinant or naturally occurring. The protein can be isolated, present in a fluid, membrane, cell or tissue sample, expressed in a cell, expressed in a membrane derived from a cell, or expressed in a whole animal. The assay can be *in vitro*, e.g., isolated protein (recombinant or naturally occurring), membranes, tissues, fluids, or cells, in an aqueous or solid phase or a combination of both, or *in vivo* in a whole animal.

Modulation is tested using one of the *in vitro* or *in vivo* assays described herein. Samples or non-human animals that are treated with a potential RDGC phosphatase inhibitor or activator are compared to control samples or animals without the test compound, to examine the extent of modulation. Control samples or animals (untreated with activators or inhibitors) are assigned a relative RDGC phosphatase activity value of 100. Inhibition of RDGC is achieved when the RDGC phosphatase activity value relative to the control is about 90%, preferably 50%, more preferably

25%. Activation of RDGC is achieved when the RDGC phosphatase activity value relative to the control is 110%, more preferably 150%, more preferable 200% higher.

The compounds tested as modulators of RDGC phosphatase can be any small chemical compound, or a biological entity, such as a protein, sugar, nucleic acid or lipid. Typically, test compounds will be small chemical molecules and peptides. Alternatively, modulators can be genetically altered versions of RDGC phosphatase. The mimetics or modulators are added to the assays in test concentrations, by any suitable means.

When assaying for RDGC phosphatase modulators and RDGC phosphatase mimetics, the test compounds are added in test concentrations to the cell or oocyte, as described above. For example, modulators can be added to cell media in aqueous solutions or organic solvents such as DMSO, for cellular uptake. Modulators and mimetics can also be administered by injection, by fusion proteins, by liposome delivery, by viral transduction, by transfection, by expression vectors, etc.

RDGC phosphatase, and RDGC phosphatase homologs and mimetics can be administered to a cell, e.g., by transduction with an expression vector encoding RDGC phosphatase or an RDGC phosphatase homolog or mimetic; by injection of RDGC phosphatase or an RDGC phosphatase homolog or mimetic; by administering RDGC phosphatase or an RDGC phosphatase homolog or mimetic in a liposome; by creating a targeted fusion protein with RDGC phosphatase or an RDGC phosphatase homolog or mimetic.

For the assays of the invention, recombinant or naturally occurring RDGC phosphatase and GPCR can be used. For example, recombinant RDGC phosphatase can be used in combination with a cell extract or membrane expresses a GPCR, or a transgenic animal can be engineered to express RDGC. Alternatively, recombinant or naturally occurring GPCRs and RDGC phosphatase or RDGC phosphatase homologs or mimetics are incubated together under standard reaction conditions, for direct detection of GPCR dephosphorylation.

Among G-protein-coupled receptors are muscarinic acetylcholine receptors (mAChR), adrenergic receptors, serotonin receptors, dopamine receptors, angiotensin receptors, adenosine receptors, bradykinin receptors, metabotropic excitatory amino acid receptors, taste cell receptors and the like. Other specific GPCRs that can be used with the invention include, but are not limited to, muscarinic receptors, *e.g.*, human M2

(GenBank accession #M16404); rat M3 (GenBank accession #M16407); human M4 (GenBank accession #M16405); human M5 (Bonner, et al., (1988) Neuron 1, pp. 403-410); and the like; neuronal nicotinic acetylcholine receptors, *e.g.*, the human α_2, α_3, and β_2, subtypes. The human α_5 subtype (Chini, et al. (1992) Proc. Natl. Acad. Sci. U.S.A. 89:1572-1576), the rat α_2 subunit (Wada, et al., (1988) Science 240, pp. 330-334); the rat α_3 subunit (Boulter, et al., (1986) Nature 319, pp. 368-374); the rat α_4 subunit (Goldman, et al. (1987) Cell 48, pp. 965-973); the rat α_5 subunit (Boulter, et al. (1990) I. Biol. Chem. 265, pp. 4472-4482); the chicken α_7 subunit (Couturier et al. (1990) Neuron 5:847-856); the rat β_2 subunit (Deneris, et al. (1988) Neuron 1, pp. 45-54) the rat β_3 subunit (Deneris, et al. (1989) J. Biol. Chem. 264, pp. 6268-6272); the rat β_4 subunit (Duvoisin, et al. (1989) Neuron 3, pp. 487-496); combinations of the rat α subunits, and s β subunits and a and p subunits; GABA receptors, e.g., the bovine x, and β_1, subunits (Schofield, et al. (1987) Nature 328, pp. 221-227); the bovine X_2, and X_3, subunits (Levitan, et al. (1988) Nature 335, pp. 76-79); the γ-subunit (Pritchett, et al. (1989) Nature 338, pp. 582-585); the β_2, and β_3, subunits (Ymer, et al. (1989) EMBO J. 8, pp. 1665-1670); the 8 subunit (Shivers, B.D. (1989) Neuron 3, pp. 327-337); and the like; glutainate receptors, e.g., rat GluR1 receptor (Hollman, et al. (1989) Nature 342, pp. 643-648); rat GluR2 and GluR3 receptors (Boulter et al. (1990) Science 249:1033-1037; rat GluR4 receptor (Keinanen et al. (1990) Science 249: 556-560); rat GluR5 receptor (Battler et al. (1990) Neuron 5:583-595); rat GluR6 receptor (Egebjerg et al. (1991) Nature 351: 745-748); rat GluR7 receptor (Battler et al. (1992) neuron 8:257-265); rat NNMAR1 receptor (Moriyoshi et al. (1991) Nature 354:31-37 and Sugihara et al. (1992) Biochem. Biophys. Res. Comm. 185:826-832); mouse NNMA el receptor (Meguro et al. (1992) Nature 357:70-74); rat NMDAR2A, NMDAR2B and NMDAR2C receptors (Monyer et al. (1992) Science 256:1217-1221); rat metabotropic mGluR1 receptor (Houamed et al. (1991) Science 252: 1318-1321); rat metabotropic mGluR2, mGluR3 and mGluR4 receptors (Tanabe et al. (1992) Neuron 8:169-179); rat metabotropic mGluR5 receptor (Abe et al. (1992) I. Biol. Chem. 267: 13361-13368); and the like; adrenergic receptors, e.g., human β1 (Frielle, et al. (1987) Proc. Natl. Acad. Sci. 84, pp. 7920-7924); human α_2 (Kobilka, et al. (1987) Science 238, pp. 650-656); hamster β_2 (Dixon, et al. (1986) Nature 321, pp. 75-79); and the like; dopamine receptors, *e.g.*, human D2 (StorTnann, et al. (1990) Molec. Pharm. 37, pp. 1-6); mammalian dopamine D2 receptor (U.S. Patent No. 5,128,254); rat (Bunzow, et al.

(1988) Nature 336, pp. 783-787); and the like; and the like; serotonin receptors, *e.g.*, human 5HT1a (Kobilka, et al. (1987) Nature 329, pp. 75-79); serotonin 5HT1C receptor (U.S. Patent No. 4,985,352); human 5HT1D (U.S. Patent No. 5,155,218); rat 5HT2 (Julius, et al. (1990) PNAS 87, pp. 928-932); rat 5HT1c (Julius, et al. (1988) Science 241, pp. 558-564), and the like.

Ion channels can also be used with the invention and include, but are not limited to, calcium channels comprised of the human calcium channel (α_2 β and/or γ-subunits (see also, W089/09834; human neuronal α_2 subunit); rabbit skeletal muscle al subunit (Tanabe, et al. (1987) Nature 328, pp. 313-E318); rabbit skeletal muscle α_2 subunit (Ellis, et al. (1988) Science 241, pp. 1661-1664); rabbit skeletal muscle p subunit (Ruth, et al. (1989) Science 245, pp. 1115-1118); rabbit skeletal muscle γ subunit (Jay, et al. (1990) Science 248, pp. 490-492); and the like; potassium ion channels, e.g., rat brain (BK2) (McKinnon, D. (1989) J. Biol Chem. 264, pp. 9230-8236); mouse brain (BK1) (Tempel, et al. (1988) Nature 332, pp. 837-839); and the like; sodium ion channels, *e.g.*, rat brain I and II (Noda, et al. (1986) Nature 320, pp. 188-192); rat brain III (Kayano, et al. (1988) FEBS Lett. 228, pp. 187-1.94); human II (ATCC No. 59742, 59743 and Genomics 5: 204-208 (1989); chloride ion channels (Thiemann, et al. (1992), Nature 356, pp. 57-60 and Paulmichl, et al. (1992) Nature 356, pp. 238-241), and others known or developed in the art.

B. Assays

The effects of the test compounds upon the function of the phosphatase can be measured by examining any of the parameters described above. Any suitable physiological change that affects phosphatase activity can be used to assess the influence of a test compound on the phosphatases of this invention. When the functional consequences are determined using intact cells or animals, one can also measure a variety of effects such as transmitter release, hormone release, transcriptional changes to both known and uncharacterized genetic markers (e.g., northern blots), changes in cell metabolism such as cell growth or pH changes, and changes in intracellular second messengers such as Ca^{2+}, IP3 or cAMP.

RDGC phosphatase activity can be measured directly, e.g., by examining dephosphorylation of an RDGC GPCR substrate such as rhodopsin. Dephosphorylation can be measured e.g., by examining transfer of a label associated with a phosphate, or

by examining GPCR mobility. In such assays, cells, membranes, tissues, or samples comprising RDGC phosphatase and a GPCR substrate are contacted with a test compound. The samples are subjected to assays that measure dephosphorylation, e.g., ELISAs, immunoassays, and western blots are probed with specific antibodies against phosphorylated or dephosphorylated GPCRs. Optionally, samples can be probed with specific antibodies using *in situ* techniques. The assays can be performed either in an aqueous phase or in a mixture of solid and aqueous phase.

In addition, RDGC phosphatase activity can be detected by examining increased electrophoretic mobility of an RDGC. In such assays, samples are subjected to electrophoresis, and western blots are probed with a suitable antibody to detect dephosphorylated or phosphorylated GPCR. Transformed cell lines can also be used *in vitro* to identify RDGC phosphatase modulators and mimetics.

RDGC phosphatase activity can also be indirectly measured, by examining GPCR signal transduction activity. Preferred assays for G-protein coupled receptor signal transduction activity include cells that are loaded with ion or voltage sensitive dyes to report receptor and activity. Assays for determining activity of such receptors can also use known agonists and antagonists for other G-protein coupled receptors as negative or positive controls to assess activity of tested compounds. In assays for identifying modulatory compounds (e.g., agonists, antagonists), changes in the level of ions in the cytoplasm or membrane voltage will be monitored using an ion sensitive or membrane voltage fluorescent indicator, respectively. Among the ion-sensitive indicators and voltage probes that may be employed are those disclosed in the Molecular Probes 1997 Catalog. For G-protein coupled receptors, promiscuous G-proteins such as G15 and G16 can be used in the assay of choice (Wilkie *et al., PNAS USA* 88:10049-10053 (1991)). Such promiscuous G-proteins allow coupling of a wide range of receptors.

Changes in ion flux may be assessed by determining changes in polarization (i.e., electrical potential) of the cell or membrane expressing GPCR. One means to determine changes in cellular polarization is by measuring changes in current (thereby measuring changes in polarization) with voltage-clamp and patch-clamp techniques, e.g., the "cell-attached" mode, the "inside-out" mode, and the "whole cell" mode (*see, e.g.*, Ackerman *et al., New Engl. J. Med.* 336:1575-1595 (1997)). Whole cell currents are conveniently determined using the standard methodology (*see, e.g.*, Hamil *et al., PFlugers. Archiv.* 391:85 (1981). Other known assays include:

radiolabeled ion flux assays and fluorescence assays using voltage-sensitive dyes (*see, e.g.*, Vestergarrd-Bogind *et al.*, *J. Membrane Biol.* 88:67-75 (1988); Gonzales & Tsien, *Chem. Biol.* 4:269-277 (1997); Daniel *et al.*, *J. Pharmacol. Meth.* 25:185-193 (1991); Holevinsky *et al.*, *J. Membrane Biology* 137:59-70 (1994)). Generally, the compounds to be tested are present in the range from 1 pM to 100 mM.

As described above, cells expressing RDGC phosphatases may be loaded with ion or voltage sensitive dyes to report receptor or ion channel activity, such as calcium channels or N-methyl-D-aspartate (NMDA) receptors, GABA receptors, kainate/AMPA receptors, nicotinic acetylcholine receptors, sodium channels, calcium channels, potassium channels excitatory amino acid (EAA) receptors, and nicotinic acetylcholine receptors.

Receptor activation typically initiates subsequent intracellular events, e.g., increases in second messengers such as IP3, which releases intracellular stores of calcium ions. Activation of some G-protein coupled receptors stimulates the formation of inositol triphosphate (IP3) through phospholipase C-mediated hydrolysis of phosphatidylinositol (Berridge & Irvine, *Nature* 312:315-21 (1984)). IP3 in turn stimulates the release of intracellular calcium ion stores. Thus, a change in cytoplasmic calcium ion levels, or a change in second messenger levels such as IP3 can be used to assess G-protein coupled receptor function. Cells expressing such G-protein coupled receptors may exhibit increased cytoplasmic calcium levels as a result of contribution from both intracellular stores and via activation of ion channels, in which case it may be desirable although not necessary to conduct such assays in calcium-free buffer, optionally supplemented with a chelating agent such as EGTA, to distinguish fluorescence response resulting from calcium release from internal stores.

Other assays can involve determining the activity of receptors which, when activated, result in a change in the level of intracellular cyclic nucleotides, e.g., cAMP or cGMP, by activating or inhibiting enzymes such as adenylate cyclase. For example, activation of some dopamine, serotonin, metabotropic glutamate receptors and muscarinic acetylcholine receptors results in a decrease in the cAMP or cGMP levels of the cytoplasm. There are cyclic nucleotide-gated ion channels, e.g., rod photoreceptor cell channels and olfactory neuron channels that are permeable to cations upon activation by binding of cAMP or cGMP (*see, e.g.*, Altenhofen *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* 88:9868-9872 (1991) and Dhallan *et al.*, *Nature* 347:184-187 (1990)). In cases where

activation of the receptor results in a decrease in cyclic nucleotide levels, it may be preferable to expose the cells to agents that increase intracellular cyclic nucleotide levels, e.g., forskolin, prior to adding a receptor-activating compound to the cells in the assay. Cells for this type of assay can be made by co-transfection of a host cell with DNA encoding a cyclic nucleotide-grated ion channel, GPCR phosphatase and DNA encoding a receptor (e.g., certain metabotropic glutamate receptors, muscarinic acetylcholine receptors, dopamine receptors, serotonin receptors, and the like), which, when activated, causes a change in cyclic nucleotide levels in the cytoplasm.

Various methods of identifying activity of chemical with respect to a target in the presence of a GPCR phosphatase can be applied, including: ion channels (PCT publication WO 93/13423), cell surface receptors (U.S. Patents 5,401,629, and 5,436,128 and PCT Application WO 93/13423 (Akong et al) and intracellular receptors (PCT publication WO 96/41013, U.S. Patent 5,548,063, U.S. Patent 5,171,671, U.S. Patent 5,274,077, U.S. Patent 4,981,784, EP 0 540 065 A1, U.S. Patent 5,071,773, and U.S. 5,298,429).

C. RDGC phosphatase modulators, homologs, and mimetics

New chemical or recombinant RDGC phosphatase mimetics, homologs, and modulators are generated by identifying compounds with RDGC phosphatase activity or the ability to modulate RDGC phosphatase activity, using the assays described above. These compounds are often referred to as lead compounds. Once a lead compound is identified, variants are typically created and evaluated for use as a therapeutic agent. An example of an RDGC phosphatase mimetic is a small peptide containing the RDGC phosphatase active site, or a small chemical molecule that has the same chemical structure as the RDGC phosphatase active site. An example of an RDGC phosphatase homolog is a naturally-occurring or recombinant variant of RDGC phosphatase that has increased stability or increased activity. An example of an RDGC phosphatase modulator is a small chemical or peptide that inhibits or activates RDGC phosphatase activity.

A wide variety of RDGC phosphatase homologs can be tested for RDGC phosphatase activity, e.g., conservative modifications, truncations, targeted fusion proteins, etc. Such molecules are typically isolated from naturally occurring sources or made using standard recombinant technology, described below, or designed by computer

assisted drug design, described below. For example, the RDGC phosphatase active site can be identified using computer assisted drug design, by site-directed mutagenesis of conserved RDGC phosphatase domains, by screening nested deletions or by screening linker scanner deletions of RDGC phosphatase. The RDGC phosphatase active site and conserved domains can also be identified by comparing the amino acid sequences of RDGC phosphatase alleles. RDGC phosphatase homologs can then be designed that include the essential components of the active site and are conservatively modified in other regions. Standard recombinant techniques are typically used to make RDGC phosphatase homologs, e.g., site-directed mutagenesis, random mutagenesis, nested deletions, linker scanner deletions, truncations, fusions, isolation of alleles, etc.

Combinatorial libraries also provide a source of potential RDGC phosphatase homologs, modulators, and mimetics, particularly mimetics and modulators. In one embodiment, a library containing a large number of potential therapeutic compounds (candidate compounds) is provided. Such "combinatorial chemical libraries" are then screened in one or more RDGC phosphatase activity assays, as described herein, to identify those library members (particular chemical species or subclasses) that display a desired characteristic activity. The compounds thus identified can serve as conventional "lead compounds" or can themselves be used as potential or actual therapeutics. A preferred method of screening for compounds is described in U.S. Serial NO. 08/858,016 to Stylie *et al.*, filed May 16, 1997.

A combinatorial chemical library is a collection of diverse chemical compounds generated by either chemical synthesis or biological synthesis, by combining a number of chemical "building blocks" such as reagents. For example, a linear combinatorial chemical library such as a polypeptide library is formed by combining a set of chemical building blocks called amino acids in every possible way for a given compound length (i.e., the number of amino acids in a polypeptide compound). Millions of chemical compounds can be synthesized through such combinatorial mixing of chemical building blocks.

Preparation and screening of combinatorial chemical libraries is well known to those of skill in the art. Such combinatorial chemical libraries include, but are not limited to, peptide libraries (*see, e.g.*, U.S. Patent 5,010,175, Furka, *Int. J. Pept. Prot. Res.* 37:487-493 (1991); Houghton *et al.*, *Nature* 354:84-88 (1991)). Peptide synthesis is by no means the only approach envisioned and intended for use with the

present invention. Other chemistries for generating chemical diversity libraries can also be used. Such chemistries include, but are not limited to: peptoids (PCT Publication No. WO 91/19735), encoded peptides (PCT Publication WO 93/20242), random bio-oligomers (PCT Publication No. WO 92/00091), benzodiazepines (U.S. Pat. No. 5,288,514), diversomers such as hydantoins, benzodiazepines and dipeptides (Hobbs *et al., Proc. Nat. Acad. Sci. USA* 90:6909-6913 (1993)), vinylogous polypeptides (Hagihara *et al., J. Amer. Chem. Soc.* 114:6568 (1992)), nonpeptidal peptidomimetics with a β-D-glucose scaffolding (Hirschmann *et al., J. Amer. Chem. Soc.* 114:9217-9218 (1992)), analogous organic syntheses of small compound libraries (Chen *et al., J. Amer. Chem. Soc.* 116:2661 (1994)), oligocarbamates (Cho *et al., Science* 261:1303 (1993)), and/or peptidyl phosphonates (Campbell *et al., J. Org. Chem.* 59:658 (1994)), nucleic acid libraries, peptide nucleic acid libraries (*see, e.g.,* U.S. Patent 5,539,083), antibody libraries (*see, e.g.,* Vaughn *et al., Nature Biotechnology*, 14(3):309-314 (1996) and PCT/US96/10287), carbohydrate libraries (*see, e.g.,* Liang *et al., Science*, 274:1520-1522 (1996) and U.S. Patent 5,593,853), small organic molecule libraries (*see, e.g.,* benzodiazepines, Baum C&EN, Jan 18, page 33 (1993); isoprenoids, U.S. Patent 5,569,588; thiazolidinones and metathiazanones, U.S. Patent 5,549,974; pyrrolidines, U.S. Patents 5,525,735 and 5,519,134; morpholino compounds, U.S. Patent 5,506,337; benzodiazepines, 5,288,514, and the like).

Devices for the preparation of combinatorial libraries are commercially available (*see, e.g.,* 357 MPS, 390 MPS, Advanced Chem Tech, Louisville KY, Symphony, Rainin, Woburn, MA, 433A Applied Biosystems, Foster City, CA, 9050 Plus, Millipore, Bedford, MA).

A number of well known robotic systems have also been developed for solution phase chemistries. These systems include automated workstations like the automated synthesis apparatus developed by Takeda Chemical Industries, LTD. (Osaka, Japan) and many robotic systems utilizing robotic arms (Zymate II, Zymark Corporation, Hopkinton, Mass.; Orca, Hewlett-Packard, Palo Alto, Calif.) which mimic the manual synthetic operations performed by a chemist. Any of the above devices are suitable for use with the present invention. The nature and implementation of modifications to these devices (if any) so that they can operate as discussed herein will be apparent to persons skilled in the relevant art. In addition, numerous combinatorial libraries are themselves commercially available (*see, e.g.,* ComGenex, Princeton, N.J., Asinex, Moscow, Ru,

Tripos, Inc., St. Louis, MO, ChemStar, Ltd, Moscow, RU, 3D Pharmaceuticals, Exton, PA, Martek Biosciences, Columbia, MD, *etc.*).

D. High throughput methodology

Any of the assays for compounds that modulate or mimic RDGC phosphatase activity, described herein, are amenable for use in high throughput screening. High throughput assays for the activity of a particular product, e.g., RDGC phosphatase or RDGC phosphatase mimetics or homologs, are well known to those of skill in the art. In addition, high throughput screening systems are commercially available (*see, e.g.*, Zymark Corp., Hopkinton, MA; Air Technical Industries, Mentor, OH; Beckman Instruments, Inc. Fullerton, CA; Precision Systems, Inc., Natick, MA, *etc.*). These systems typically automate entire procedures including all sample and reagent pipetting, liquid dispensing, timed incubations, and final readings of the microplate in detector(s) appropriate for the assay. These configurable systems provide high thruput and rapid start up as well as a high degree of flexibility and customization. The manufacturers of such systems provide detailed protocols the various high throughput. Thus, for example, Zymark Corp. provides technical bulletins describing screening systems for detecting the modulation of gene transcription, ligand binding, and the like.

Such high throughput assays often incorporate solid substrates such as a membrane (e.g., nitrocellulose or nylon), a microtiter dish (e.g., PVC, polypropylene, or polystyrene), a test tube (glass or plastic), a dipstick (e.g., glass, PVC, polypropylene, polystyrene, latex, and the like), a microcentrifuge tube, or a glass, silica, plastic, metallic or polymer bead or other substrate such as paper.

Often in the assays of the invention, a molecule such as a GPCR is labeled with a detectable moiety. For example, in electrophoretic mobility assays, a GPCR can be labeled at the C-terminus or N-terminus.

The particular label or detectable group used in the assay is not a critical aspect of the invention, as long as it does not significantly interfere with RDGC phosphatase activity. Such detectable labels have been well-developed in the field of immunoassays and, in general, most any label useful in such methods can be applied to the present invention. A wide variety of labels may be used, with the choice of label depending on sensitivity required, ease of conjugation with the compound, stability

requirements, available instrumentation, and disposal provisions. A preferred label is a fluorescent label.

Suitable labels are any composition detectable by spectroscopic, photochemical, biochemical, immunochemical, electrical, optical or chemical means. Useful labels in the present invention include magnetic beads (e.g., DYNABEADS™), fluorescent dyes and molecules (e.g., fluorescein isothiocyanate, Texas red, rhodamine, and the like), radiolabels (e.g., ^{3}H, ^{125}I, ^{35}S, ^{14}C, or ^{32}P), enzymes (e.g., horse radish peroxidase, alkaline phosphatase and others commonly used in an ELISA), and colorimetric labels such as colloidal gold or colored glass or plastic beads (e.g., polystyrene, polypropylene, latex, etc.). Preferred labels include fluorescent molecules and dyes, Green Fluorescent Protein (GFP; see, e.g., U.S. 5,625048; WO96/23810; WO97/28261; PCT/UX97/12410; PCT/US97/14593), β-lactamase (see, e.g., U.S. 5,741,657; and WO96/30540), fluorescent membrane potential indicators, also known as fluorescent membrane voltage sensors (see, e.g., U.S. 5,661,035 and WO96/41166), and FRET (fluorescence resonance energy).

The label may be coupled directly or indirectly to the desired component of the assay according to methods well known in the art. Non-radioactive labels are often attached by indirect means. For example, a ligand molecule (e.g., biotin) is covalently bound to the molecule. The ligand then binds to another molecules (e.g., streptavidin) molecule, which is either inherently detectable or covalently bound to a signal system, such as a detectable enzyme, a fluorescent compound, or a chemiluminescent compound. The ligands and their targets can be used in any suitable combination with antibodies that recognize the RDGC phosphatase substrate, or secondary antibodiés that recognize anti-RDGC phosphatase-substrates.

The molecules can also be conjugated directly to signal generating compounds, e.g., by conjugation with an enzyme or fluorophore. Enzymes of interest as labels will primarily be hydrolases, particularly phosphatases, esterases and glycosidases, or oxidotases, particularly peroxidases. Fluorescent compounds include fluorescein and its derivatives, rhodamine and its derivatives, dansyl, umbelliferone, etc. Chemiluminescent compounds include luciferin, and 2,3-dihydrophthalazinediones, e.g., luminol. For a review of various labeling or signal producing systems that may be used, see U.S. Patent No. 4,391,904.

Means of detecting labels are well known to those of skill in the art. Thus, for example, where the label is a radioactive label, means for detection include a scintillation counter or photographic film as in autoradiography. Where the label is a fluorescent label, it may be detected by exciting the fluorochrome with the appropriate wavelength of light and detecting the resulting fluorescence. The fluorescence may be detected visually, by means of photographic film, by the use of electronic detectors such as charge coupled devices (CCDs) or photomultipliers and the like. Similarly, enzymatic labels may be detected by providing the appropriate substrates for the enzyme and detecting the resulting reaction product. Finally simple colorimetric labels may be detected simply by observing the color associated with the label. Thus, in various dipstick assays, conjugated gold often appears pink, while various conjugated beads appear the color of the bead.

E. Computer assisted drug design

Yet another assay for compounds that modulate RDGC phosphatase activity involves computer assisted drug design, in which a computer system is used to generate a three-dimensional structure of a protein based on the structural information encoded by the amino acid sequence. The three dimensional structure of the protein is then used to identify potential ligands that bind to the protein, or to identify molecules that are mimetics of the protein of interest. For example, the three-dimensional structure of RDGC phosphatase can be used to identify RDGC phosphatase mimetics and modulators that bind to RDGC phosphatase. The structure can also be used to identify the RDGC phosphatase protease active site. Similarly, the three-dimensional structure of GPCRs such as rhodopsin can be used to identify RDGC phosphatase mimetics that bind to GPCRs, or RDGC phosphatase modulators that bind to GPCRs. In the computer system, the input amino acid sequence interacts directly and actively with a preestablished algorithm in a computer program to yield secondary, tertiary, and quaternary structural models of the protein. The models of the protein structure are then examined to identify regions of the structure that have the ability to bind, e.g., RDGC phosphatase or a GPCR. These regions are then used to identify ligands that bind to the protein of interest, or regions where RDGC phosphatase interacts with the GPCR, or the regions where GPCR interacts with RDGC phosphatase.

The three-dimensional structural model of the protein is generated by entering channel protein amino acid sequences of at least 10 amino acid residues or corresponding nucleic acid sequences encoding RDGC phosphatase or GPCR into the computer system. The amino acid sequence represents the primary sequence or subsequence of the protein, which encodes the structural information of the protein. The sequence is entered into the computer system from computer keyboards, computer readable substrates that include, but are not limited to, electronic storage media (e.g., magnetic diskettes, tapes, cartridges, and chips), optical media (e.g., CD ROM), information distributed by internet sites, and by RAM. The three-dimensional structural model of RDGC phosphatase or GPCR is then generated by the interaction of the amino acid sequence and the computer system, using software known to those of skill in the art.

The amino acid sequence represents a primary structure that encodes the information necessary to form the secondary and tertiary structure of RDGC phosphatase or GPCR. The software looks at certain parameters encoded by the primary sequence to generate the structural model. These parameters are referred to as "energy terms," and primarily include electrostatic potentials, hydrophobic potentials, solvent accessible surfaces, and hydrogen bonding. Secondary energy terms include van der Waals potentials. Biological molecules form the structures that minimize the energy terms in a cumulative fashion. The computer program is therefore using these terms encoded by the primary structure or amino acid sequence to create the secondary structural model.

The tertiary structure of the protein encoded by the secondary structure is then formed on the basis of the energy terms of the secondary structure. The user at this point can enter additional variables such as whether the protein is membrane bound or soluble, its location in the body, and its cellular location, e.g., cytoplasmic, surface, or nuclear. These variables along with the energy terms of the secondary structure are used to form the model of the tertiary structure. In modeling the tertiary structure, the computer program matches hydrophobic faces of secondary structure with like, and hydrophilic faces of secondary structure with like.

Once the structure has been generated, potential ligand binding regions are identified by the computer system. Three-dimensional structures for potential ligands are generated by entering amino acid or nucleotide sequences or chemical formulas of compounds, as described above. The three-dimensional structure of the potential ligand is then compared to that of the RDGC phosphatase or GPCR protein to identify ligands

that bind to RDGC phosphatase or GPCR. Binding affinity between the protein and ligands is determined using energy terms to determine which ligands have an enhanced probability of binding to the protein.

IV. How to make recombinant RDGC phosphatase and GPCR proteins

As described above, naturally occurring or recombinant RDGC phosphatase and GPCRs and homologs and mimetics thereof can be used in the assays of the invention. Recombinant RDGC phosphatase and GPCRs are conveniently used for *in vitro* and *in vivo* assays. In addition, recombinant RDGC phosphatase homologs and fusion proteins can be prepared for testing as RDGC phosphatase mimetics and potential therapeutic agents. The preparation of recombinant RDGC phosphatase and GPCRs is described below, as well as methods for isolating naturally occurring proteins.

A. General recombinant DNA methods

Often, recombinant proteins are used in the assays of the present invention, e.g., recombinant RDGC phosphatase, GPCRs, mutants thereof, and functional equivalents. For producing recombinant proteins, this invention relies on routine techniques in the field of recombinant genetics. Basic texts disclosing the general methods of use in this invention include Sambrook *et al.*, *Molecular Cloning, A Laboratory Manual* (2nd ed. 1989); Kriegler, *Gene Transfer and Expression: A Laboratory Manual* (1990); and *Current Protocols in Molecular Biology* (Ausubel *et al.*, eds., 1994)).

For nucleic acids, sizes are given in either kilobases (kb) or base pairs (bp). These are estimates derived from agarose or acrylamide gel electrophoresis, from sequenced nucleic acids, or from published DNA sequences. For proteins, sizes are given in kilodaltons (kDa) or amino acid residue numbers. Proteins sizes are estimated from gel electrophoresis, from sequenced proteins, from derived amino acid sequences, or from published protein sequences.

Oligonucleotides that are not commercially available can be chemically synthesized according to the solid phase phosphoramidite triester method first described by Beaucage & Caruthers, *Tetrahedron Letts.* 22:1859-1862 (1981), using an automated synthesizer, as described in Van Devanter *et. al.*, *Nucleic Acids Res.* 12:6159-6168 (1984). Purification of oligonucleotides is by either native acrylamide gel electrophoresis

or by anion-exchange HPLC as described in Pearson & Reanier, *J. Chrom.* 255:137-149 (1983).

The sequence of the cloned genes and synthetic oligonucleotides can be verified after cloning using, e.g., the chain termination method for sequencing double-stranded templates of Wallace *et al., Gene* 16:21-26 (1981).

B. Cloning methods for the isolation of nucleotide sequences encoding RDGC phosphatase and GPCRs

In general, the nucleic acid sequences encoding RDGC phosphatase, GPCRs and related nucleic acid sequence homologs are cloned from cDNA and genomic DNA libraries or isolated using amplification techniques with oligonucleotide primers. For example, RDGC phosphatase sequences are typically isolated from *Drosophila* nucleic acid (genomic or cDNA) libraries, while genes for GPCRs (e.g., rhodopsin, etc.) can be cloned from mammalian libraries, preferably human libraries.

Amplification techniques using primers can also be used to amplify and isolate RDGC phosphatase and GPCRs from DNA or RNA (*see* U.S. Patents 4,683,195 and 4,683,202; *PCR Protocols: A Guide to Methods and Applications* (Innis *et al.*, eds, 1990)). Methods such as polymerase chain reaction (PCR) and ligase chain reaction (LCR) can be used to amplify nucleic acid sequences of RDGC phosphatase directly from mRNA, from cDNA, from genomic libraries or cDNA libraries, and from plasmids. Degenerate oligonucleotides can be designed to amplify homologs. These primers can be used, e.g., to amplify a probe of several hundred nucleotides, which is then used to screen a human library for full-length RDGC phosphatase. Alternatively, the nucleic acid for RDGC phosphatase can be directly amplified. Similar procedures can be used to isolate sequences encoding GPCRs.

Nucleic acids encoding RDGC phosphatase and GPCRs can also be isolated from expression libraries using antibodies as probes. Such polyclonal or monoclonal antibodies can be raised using recombinant or naturally occurring RDGC phosphatase or GPCRs as antigens.

Synthetic oligonucleotides can be used to construct recombinant RDGC phosphatase genes for use as probes, for expression of protein, and for construction of polymorphic variants or mutants such as deletion mutants. This method is performed using a series of overlapping oligonucleotides usually 40-120 bp in length, representing

both the sense and nonsense strands of the gene. These DNA fragments are then annealed, ligated and cloned.

Polymorphic variants, alleles, and interspecies homologs that are substantially identical to RDGC phosphatase or GPCRs can be isolated using RDGC phosphatase and GPCR nucleic acid probes and oligonucleotides under stringent hybridization conditions, by screening libraries using probes, or using amplification techniques as described above. Alternatively, expression libraries can be used to clone polymorphic variants, alleles, and interspecies homologs, by detecting expressed homologs immunologically with antisera or purified antibodies, which also recognize and selectively bind to the homolog. The gene encoding *Drosophila* RDGC phosphatase has been cloned and sequenced (Steele *et al., supra*)

The nucleic acids of interest are typically cloned into intermediate vectors before transformation into prokaryotic or eukaryotic cells for replication and/or expression. These intermediate vectors are typically prokaryote vectors, e.g., plasmids, or shuttle vectors, as described below.

C. Expression in prokaryotes and eukaryotes

To obtain high level expression of a cloned gene, such as those cDNAs encoding RDGC phosphatase and GPCRs, one typically subclones the nucleic acid into an expression vector that contains a strong promoter to direct transcription, a transcription/translation terminator, and if for a nucleic acid encoding a protein, a ribosome binding site for translational initiation. Suitable bacterial promoters are well known in the art and described, e.g., in Sambrook *et al.* and Ausubel *et al.* Bacterial expression systems for expressing the RDGC phosphatase protein are available in, e.g., *E. coli, Bacillus sp.*, and *Salmonella* (Palva *et al.*, *Gene* 22:229-235 (1983); Mosbach *et al.*, *Nature* 302:543-545 (1983)). Kits for such expression systems are commercially available. Eukaryotic expression systems for mammalian cells, yeast, and insect cells are well known in the art and are also commercially available.

The promoter used to direct expression of a heterologous nucleic acid depends on the particular application and is not critical. Exemplary promoters include the SV40 early promoter, SV40 later promoter, metallothionein promoter, murine mammary tumor virus promoter, Rous sarcoma virus promoter, polyhedrin promoter, or other promoters shown effective for expression in eukaryotic cells, as well as prokaryotic

promoters. The promoter is preferably positioned about the same distance from the heterologous transcription start site as it is from the transcription start site in its natural setting. As is known in the art, however, some variation in this distance can be accommodated without loss of promoter function.

In addition to the promoter, the expression vector typically contains a transcription unit or expression cassette that contains all the additional elements required for the expression of the nucleic acid in host cells. A typical expression cassette thus also contains signals required for efficient polyadenylation of the transcript, ribosome binding sites, and translation termination. The nucleic acid sequence encoding the gene of choice may typically be linked to a cleavable signal peptide sequence to promote secretion of the encoded protein by the transformed cell. Such signal peptides would include, among others, the signal peptides from tissue plasminogen activator, insulin, and neuron growth factor, and juvenile hormone esterase of *Heliothis virescens*. Additional elements of the cassette may include enhancers and, if genomic DNA is used as the structural gene, introns with functional splice donor and acceptor sites.

In addition to a promoter sequence, the expression cassette should also contain a transcription termination region downstream of the structural gene to provide for efficient termination. The termination region may be obtained from the same gene as the promoter sequence or may be obtained from different genes.

Additional elements that are typically included in expression vectors also include a replicon that functions in *E. coli*, a gene encoding antibiotic resistance to permit selection of bacteria that harbor recombinant plasmids, unique restriction sites in nonessential regions of the plasmid to allow insertion of eukaryotic sequences. In addition, some expression systems have markers that provide gene amplification such as thymidine kinase, hygromycin B phosphotransferase, and dihydrofolate reductase. The particular antibiotic resistance gene chosen is not critical, any of the many resistance genes known in the art are suitable. The prokaryotic sequences are preferably chosen such that they do not interfere with the replication of the DNA in eukaryotic cells, if necessary.

The particular expression vector used to transport the genetic information into the cell is not particularly critical. Any of the conventional vectors used for expression in eukaryotic or prokaryotic cells may be used. Standard bacterial expression vectors include plasmids such as pBR322 based plasmids, pSKF, pET23D, and fusion

expression systems such as GST and LacZ. Other exemplary eukaryotic vectors include pMSG, pAV009/A$^+$, pMTO10/A$^+$, pMAMneo-5, baculovirus pDSVE. Tags can also be added to recombinant proteins to provide convenient methods of isolation, e.g., c-myc, or hexahistidine.

Standard transfection methods are used to produce bacterial, mammalian, yeast or insect cell lines that express large quantities of RDGC phosphatase protein, which are then purified using standard techniques (*see, e.g.*, Colley *et al.*, *J. Biol. Chem.* 264:17619-17622 (1989); *Guide to Protein Purification*, in *Methods in Enzymology*, vol. 182 (Deutscher, ed., 1990)). Transformation of eukaryotic and prokaryotic cells are performed according to standard techniques, e.g., calcium phosphate transfection, polybrene, protoplast fusion, electroporation, liposomes, microinjection, plasma vectors, viral vectors (*see, e.g.*, Morrison, *J. Bact.* 132:349-351 (1977); Clark-Curtiss & Curtiss, *Methods in Enzymology* 101:347-362 (Wu *et al.*, eds, 1983).

After the expression vector is introduced into the cells, the transfected cells are cultured under conditions favoring expression of the gene of choice, which is recovered from the culture using standard techniques identified below.

V. Purification of RDGC phosphatase and GPCRs and cellular expression

Naturally occurring or recombinant RDGC phosphatase and GPCRs can be purified for use in the assays of the invention. Naturally occurring RDGC phosphatase is purified, e.g., from *Drosophila*. Naturally occurring GPCRs are purified from a variety of knowbn sources. Recombinant RDGC phosphatase and GPCR are purified from any suitable expression system.

RDGC phosphatase and GPCRs may be purified to substantial purity by standard techniques, including selective precipitation with such substances as ammonium sulfate; column chromatography, immunopurification methods, and others (*see*, *e.g.*, Scopes, *Protein Purification: Principles and Practice* (1982); U.S. Patent No. 4,673,641; Ausubel *et al.*, *supra*; and Sambrook *et al.*, *supra*).

A number of procedures can be employed when recombinant proteins are purified. For example, proteins having established molecular adhesion properties can be reversible fused to the protein of choice. With the appropriate ligand, the protein can be selectively adsorbed to a purification column and then freed from the column in a

relatively pure form. The fused protein is then removed by enzymatic activity. Finally, the protein of choice can be purified using affinity or immunoaffinity columns.

A. Purification of protein from recombinant bacteria

Recombinant proteins are expressed by transformed bacteria in large amounts, typically after promoter induction; but expression can be constitutive. Promoter induction with IPTG is a one example of an inducible promoter system. Bacteria are grown according to standard procedures in the art. Fresh or frozen bacteria cells are used for isolation of protein.

Proteins expressed in bacteria may form insoluble aggregates ("inclusion bodies"). Several protocols are suitable for purification of recombinant inclusion bodies. For example, purification of inclusion bodies typically involves the extraction, separation and/or purification of inclusion bodies by disruption of bacterial cells, e.g., by incubation in a buffer of 50 mM TRIS/HCL pH 7.5, 50 mM NaCl, 5 mM $MgCl_2$, 1 mM DTT, 0.1 mM ATP, and 1 mM PMSF. The cell suspension can be lysed using 2-3 passages through a French Press, homogenized using a Polytron (Brinkman Instruments) or sonicated on ice. Alternate methods of lysing bacteria are apparent to those of skill in the art (*see, e.g.*, Sambrook *et al., supra*; Ausubel *et al., supra*).

If necessary, the inclusion bodies are solubilized, and the lysed cell suspension is typically centrifuged to remove unwanted insoluble matter. Proteins that formed the inclusion bodies may be renatured by dilution or dialysis with a compatible buffer. Suitable solvents include, but are not limited to urea (from about 4 M to about 8 M), formamide (at least about 80%, volume/volume basis), and guanidine hydrochloride (from about 4 M to about 8 M). Other suitable buffers are known to those skilled in the art. The protein of choice is separated from other bacterial proteins by standard separation techniques, e.g., with Ni-NTA agarose resin.

Alternatively, it is possible to purify the recombinant protein from bacteria periplasm. After lysis of the bacteria, when the recombinant protein is exported into the periplasm of the bacteria, the periplasmic fraction of the bacteria can be isolated by cold osmotic shock in addition to other methods known to skill in the art. To isolate recombinant proteins from the periplasm, the bacteria are centrifuged and the pellet is resuspended in ice-cold 5 mM $MgSO_4$ and kept in an ice bath for approximately 10 minutes, for cell lysis to occur. The cell suspension is centrifuged and the supernatant

decanted and saved. The recombinant proteins present in the supernatant can be separated from the host proteins by standard separation techniques well known to those of skill in the art.

B. Standard protein separation techniques for purifying recombinant and naturally occurring proteins

Solubility fractionation

Often as an initial step, particularly if the protein mixture is complex, an initial salt fractionation can separate many of the unwanted host cell proteins (or proteins derived from the cell culture media) from the protein of interest. The preferred salt is ammonium sulfate. Ammonium sulfate precipitates proteins by effectively reducing the amount of water in the protein mixture. Proteins then precipitate on the basis of their solubility. The more hydrophobic a protein is, the more likely it is to precipitate at lower ammonium sulfate concentrations. A typical protocol includes adding saturated ammonium sulfate to a protein solution so that the resultant ammonium sulfate concentration is between 20-30%. This concentration will precipitate the most hydrophobic of proteins. The precipitate is then discarded (unless the protein of interest is hydrophobic) and ammonium sulfate is added to the supernatant to a concentration known to precipitate the protein of interest. The precipitate is then solubilized in buffer and the excess salt removed if necessary, either through dialysis or diafiltration. Other methods that rely on solubility of proteins, such as cold ethanol precipitation, are well known to those of skill in the art and can be used to fractionate complex protein mixtures.

Size differential filtration

The molecular weight of the protein of choice can be used to isolated it from proteins of greater and lesser size using ultrafiltration through membranes of different pore size (for example, Amicon or Millipore membranes). As a first step, the protein mixture is ultrafiltered through a membrane with a pore size that has a lower molecular weight cut-off than the molecular weight of the protein of interest. The retentate of the ultrafiltration is then ultrafiltered against a membrane with a molecular cut off greater than the molecular weight of the protein of interest. The recombinant

protein will pass through the membrane into the filtrate. The filtrate can then be chromatographed as described below.

Column chromatography

The protein of choice can also be separated from other proteins on the basis of its size, net surface charge, hydrophobicity, and affinity for ligands. In addition, antibodies raised against recombinant or naturally occurring proteins can be conjugated to column matrices and the proteins immunopurified. All of these methods are well known in the art. It will be apparent to one of skill that chromatographic techniques can be performed at any scale and using equipment from many different manufacturers (e.g., Pharmacia Biotech). For example, RDGC phosphatase can be purified using a PA63 heptamer affinity column (Singh *et al., J. Biol. Chem.* 269:29039-29046 (1994)).

VI. Kits

The present invention also provides for kits for screening for modulators of RDGC phosphatase. Such kits can be prepared from readily available materials and reagents. For example, such kits can comprise any one or more of the following materials: biologically active RDGC phosphatase, reaction tubes, and instructions for testing RDGC phosphatase activity. A wide variety of kits and components can be prepared according to the present invention, depending upon the intended user of the kit and the particular needs of the user. For example, the kit can be tailored for *in vivo* or *in vitro* phosphorylation of a GPCR such as rhodopsin, determination of electrophoretic mobility, or GPCR signal transduction activity.

All publications and patent applications cited in this specification are herein incorporated by reference as if each individual publication or patent application were specifically and individually indicated to be incorporated by reference.

Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, it will be readily apparent to one of ordinary skill in the art in light of the teachings of this invention that certain changes and modifications may be made thereto without departing from the spirit or scope of the appended claims.

EXAMPLES

The following examples are provided by way of illustration only and not by way of limitation. Those of skill in the art will readily recognize a variety of noncritical parameters that could be changed or modified to yield essentially similar results.

Example 1

RDGC was analyzed biochemically, physiologically, and genetically to determine its activity as a GPCR phosphatase (see Vinos *et al., Science* 277:687-690 (1997, herein incorporated by reference). First, light dependent phosphorylation of rhodopsin in wild-type and *rdgC* mutant photoreceptor neurons were examined.

The following stocks were used in the phosphorylation experiments: wl, w1118;; ninaE234, w1118;; rdgC306 cu red, w1118;; P[ninaED356 ry+] ry ninaEI17 es (Rh1D356), w1118; P[ninaED356 ry+]; rdgC306 cu red ry ninaEI17 es (Rh1D356; rdgC). The amount of label ingested was monitored by adding a green food colorant to the radioactive food. For each sample, 10 flies were subjected to 4 hour starvation in the dark and then transferred to a vial containing 50 μl of 1% sucrose, 0.5% agarose and 50 μCi of carrier-free ^{32}PO4. Flies were kept in a dark, humid chamber overnight and then exposed to either 15 min or 20 s of blue or orange light. Light exposure times were selected to maximize differences between phenotypes. Flies were then frozen in liquid nitrogen and dried in cold acetone for 3 hours at -80°C followed by 16 hours at -20°C. Retinas were hand-dissected and homogenized in electrophoresis sample buffer containing the phosphatase inhibitors okadaic acid and microcystin. Proteins were separated by denaturing electrophoresis, blotted to nitrocellulose, analyzed on a phosphoimager, and then probed with specific antibodies.

By using blue or orange light, it was possible to experimentally shift between the active and inactive states of the light receptor molecule. Control and mutant flies were fed $^{32}P0_4$ for 16 hr in the dark, then illuminated with intense light, frozen in liquid nitrogen, retinas were dissected and the in vivo-phosphorylated retinal proteins were separated by SDS-PAGE. Separated retinal proteins were also transferred to nitrocellulose and probed with anti-rhodopsin antibodies. Rh1 is phosphorylated in a blue-light dependent manner, while subsequent exposure to orange light promotes its dephosphorylation (Fig. 1A; Fig. 1B, lanes 1 and 2). As expected, Rh1 is missing in

ninaE mutants (lane 3). Remarkably, Rhl is dramatically hyper-phosphorylated in rdgC mutants, and remains in this state even after exposure to orange light (lane 4). This is consistent with the loss of a major rhodopsin phosphatase activity in rdgC mutant photoreceptor cells. No other proteins were found to have altered phosphorylation profiles in rdgC.

In vivo phosphorylation of retinal proteins is represented in FIGURE 1. Figure 1(A) shows an autoradiogram of SDS-polyacrylamide gel electrophoresis (PAGE) of $^{32}PO_4$ *in vivo* labeled retinal proteins from flies that were exposed to blue light (B) or orange light (O) for 15 min. Blue light promoted phosphorylation of Rhl, whereas orange light promoted dephosphorylation (W, white-eyed control flies).

Next, transgenic flies were generated that express a truncated rhodopsin molecule, Rh1Δ356. This mutation eliminates the last 18 amino acid residues of rhodopsin, including the serines and threonines in the cytoplasmic tail. These residues are phosphorylated by GRKs. This truncated molecule was expressed in a *ninaE* mutant background such that the only rhodopsin present in photoreceptors was the one directed by the transgene. The truncated receptor was expressed in near normal amounts and the cells displayed normal light response. Rhodopsin was not hyperphosphorylated in Rh1Δ356 flies (Fig. 1B).

In Figure 1(B) the experiment in (A) was repeated with a 20 s pulse of light to quantitatively examine the *rdg*C phenotype. Upper panel; Autoradiogam of SDS-PAGE of $^{32}PO_4$ *in vivo* labeled retinal proteins. B denotes flies exposed to 20 s of blue light; BO denotes flies exposed to 20 s of blue light followed by 20 s of orange light. *NinaE* represents a null mutation in the structural gene for Rh1 *rdg*C is a mutation in the RDGC phosphatase gene, and Rh1Δ356 is the truncation of the last 18 residues of the COOH-terminal tail of rhodopsin. The results are representative of three independent experiments. Lower panel: The same gel blotted and probed with antibodies to Rh1. The truncation of rhodopsin results in a faster migrating polypeptide.

Figure 1(C) shows a histogram of the relative extent of ^{32}P incorporation into Rh1. Samples were normalized to control flies exposed to blue and orange light sequentially (BO) and corrected for the amount of rhodopsin loaded. Data are means = SEM of triplicate determinations.

Example 2

The *rdg*C phenotype was examined by microscopy. FIGURE 2 indicates that Rh1Δ356 suppresses *rdg*C light-dependent retinal degeneration. For microscopy, flies were exposed to white light [30 W white lamp, attenuated 100 fold, at 15 cm] for six days. Fly heads were cut, fixed and embedded in resin as described in Smith *et al.*, *Science* 254:1478-84 (1991). 1 μm thick sections were stained with methylene blue/borax prior to analysis. Shown are cross sections (1 μm thick) through adult retinas after 6 days of light exposure.

Figure 2(A) shows control flies with normal retinal morphology, with ommatidial clusters organized as a patterned array. Figure 2(B) shows retinas from *rdg*C flies show marked degeneration. Figure 2(C) shows dgq mutants that lack the ∝ subunit of the G protein that couples rhodopsin to PLC do not show light-dependent degeneration. In Figure 2(D), dgq is unable to suppress the degeneration of the Dgq; *rdg*C double mutants; in figure 2(E) Rh1Δ356 transgenic flies display normal retinal morphology; in figure 2(F), Rh1Δ356 suppresses *rdg*C degeneration in Rh1Δ356; *rdg*C double mutants. If kept in the dark, all genotypes display normal retinal morphology. Scale bar, 40 μm.

Example 3

In wild-type photoreceptor cells, termination of the light response results from the concerted action of regulatory events at multiple steps. Because *rdg*C mutants accumulate phosphorylated rhodopsin, alterations in the kinetics of photoreceptor cell inactivation were examined. Examination of photoreceptor light responses by electroretinograms (ERGs) and intracellular recordings showed that the *rdg*C mutant exhibited a notable decrease in the rate of deactivation relative to control flies.

Electroretinograms (ERGs) of 4 days old adult, dark-raised flies were recorded. Stimulating light was delivered with a 450 W Xenon lamp (Osram) and filtered with either 480 nm bandpass (Oriel, 53850) filter for blue light or 570 nm longpass (Oriel, 51310) filter for orange light. Recordings in Fig. 3A were performed with orange light attenuated 1585 fold. For intracellular recordings, a coronal section was made through the compound eye. Dorsal hemispheres of the head were mounted upside down on a coverslip and immediately immersed in Schneiders medium (Gibco). Photoreceptor cells were impaled with 90-150 mV electrodes filled with 2M KC1.

Maximal differences in deactivation time are found when 0.5 s or longer stimuli are used.

Under whole cell recording conditions, no significant differences in deactivation time were found. Figure 3(A) shows that *rdg*C mutants have defective deactivation kinetics: ERGs of wt, *rdg*C, Rh7Δ356, and Rh1Δ356; *rdg*C mutant flies. Photoreceptors were given a 0.5 s pulse of orange light. *rdg*C mutants display strong defects in the deactivation kinetics (arrow), and this phenotype is suppressed by the Rh1Δ356 truncation. Traces from 15 independent measurements were averaged. Figure 3(B) shows intracellular recordings of light-activated responses from control and *rdg*C photoreceptors. This recording demonstrates the photoreceptor specificity of the phenotype (arrow). For control flies, t_{50}=34 ± 2 ms; for *rdg*C flies, t_{50} = 136 ± 22 ms (n=26). Data are means = SEM. Figure 3(C) shows a histogram of the deactivation time for the different ERG phenotypes. For control flies, t_{05} = 0.17 ±003 S; for *rdg*C flies, t_{05} = 1.8±0.3 s; for Rh1Δ356 flies, t_{85} = O.16±0.03 s; for Rh1Δ356; rdgC flies, t_{25}=0.32±0.03 s (n- 15). Data are means = SD.

Example 4

Arrestin binding is required for termination of the active state of GPCRs. In vertebrate photoreceptors, the formation of a rhodopsin-arrestin complex is largely determined by the phosphorylation state of rhodopsin. Because *rdg*C mutants accumulate hyperphosphorylated rhodopsin, the deactivation defect may result from a defect in the rhodopsin-arrestin interaction. A manifestation of this interaction *in vivo* is the prolonged depolarizing afterpotential (PDA). This sustained photoresponse occurs when a substantial amount of rhodopsin has been photoconverted from R to the active M state. A PDA results when metarhodopsin is produced in excess of free arrestin. Wild-type photoreceptors require ~20% conversion of R to M to trigger a PDA. This amount of rhodopsin isomerization approximately equals the total number of arrestin molecules in the photoreceptor cell. Mutants expressing small amounts of arrestin enter a PDA with very little light (because only a small amount of M would readily saturate arrestin availability), whereas mutants that reduce amounts of Rh1 to less than those of arrestin prevent entry into a PDA (there could never be an excess of M over arrestin). In dark raised *rdg*C mutants, the amounts of arrestin and rhodopsin are the same as in the wild-type. Like arrestin mutant photoreceptors, *rdg*C photoreceptors entered a PDA with

approximately on-eight as much light as did photoreceptors in control flies (see Fig. 4B). In contrast, Rh1Δ356; *rdg*C double mutants displayed a normal PDA. THus, RDGC is required for normal rhodopsin function. The deactivation defect may be attributable to impaired arrestin function that results from rhodopsin hyperphosphorylation.

Defects in the PDA response of rdgC flies are shown in FIGURE 4. (A) ERG recording of a white-eyed control fly showing a prototypical PDA response. O, orange light (M-R conversion); B, blue light (R-M conversion). Figure 4(B) shows a histogram of relative amount of blue light required to enter a PDA for the different genotypes. Values are normalized to control flies and represent means ±SEM of triplicate determinations. Aur2, a known mutant with a PDA defect (Dolph *et al.*, *Science* 260:1910 (1993)), is shown for comparative purposes.